CTLA4+rs231775 gene polymorphism increases PCOS, regardless of the levels of interleukin-6 and tumor necrosis factor-α in the serum

CTLA4 and increased risk of PCOS

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Abstract

Aim: Polycystic ovarian syndrome (PCOS) is a long-standing inflammation-related disease with increased levels of circulating pro-inflammatory markers. By affecting inflammatory cytokine production, cytotoxic T lymphocyte-associated antigen (CTLA-4) polymorphism can alter the immune system and trigger distinct disease states. The aim of the study was to investigate if CTLA4 polymorphism is associated with PCOS, and if so, (2) whether this situation influences serum interleukin-6 (IL-6) and TNF-alpha in PCOS.

Material and Methods: CTLA4+rs231775 gene polymorphism with IL-6 and TNF-α levels were determined in 92 PCOS women and 88 healthy controls. Study groups were further subdivided according to body mass index (BMI) and the degree of insulin resistance (IR), and comparisons were made within each study group.

Results: The prevalence of the A allele of single nucleotide polymorphism (SNP) rs231775 was more frequent in PCOS women compared with healthy controls [OR: 1.99, 95% CI:1.273-3.107, p =0.0023]. The heterozygous genotype was also shown to be strongly associated with PCOS development [OR: 3.041, 95%CI:1.604-5.766, p=0.0005]. Although TNF-α levels of PCOS patients were detected to be elevated, no difference was found in the study groups with respect to serum IL-6 levels. In addition, no association was observed between CTLA4+rs231775 polymorphism and serum pro-inflammatory cytokine levels.

Discussion: The present study demonstrates for the first time that CTLA4+rs231775 gene polymorphism increases susceptibility to PCOS 2 times more in the case of A allele carriage and 3 times more in heterozygous individuals, independent from the long-standing low-grade inflammatory disease state encountered in patients with PCOS.

Keywords

Polycystic Ovary Syndrome, CTLA4, TNF-α, IL-6, Polymorphism

Introduction

Polycystic ovary syndrome (PCOS) is a primary reason for infertility in females and is estimated to affect 5-15% of women of reproductive age. Although PCOS is considered a hormonal disorder characterized by insulin resistance and a hyperandrogenic state, growing evidence in recent years suggests that genetic abnormalities play an influential role in PCOS development by altering immune and autoimmune responses [1].

It is well established that various cytokines are critically important for the basic phases of reproduction, such as ovarian follicular development (follicle formation and activation), ovulation, fertilization, implantation of the fertilized ovum, and normal pregnancy [2]. Furthermore, it is commonly recognized that cytokines likely take part in the pathophysiology of endometriosis, PCOS, and unexplained infertility [1, 3-4]. In this sense, it has been demonstrated that the inflammatory state is important in PCOS.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) controls the activation of primary and secondary peptide-specific CD4(+) T cells and is considered a strong candidate susceptibility gene for distinct types of autoimmune and tumoral diseases [5].

The CTLA-4–ligand interplay negatively alters interleukin-2 (IL-2) production, T-cell proliferation, and cell cycle progression [5]. Furthermore, antibody-mediated CTLA-4 blockade prevents tolerance development, enhances antitumor responses, and exacerbates autoimmune diseases [6]. Accumulating evidence in recent years has demonstrated a particular CTLA-4 genetic polymorphism conferring susceptibility to numerous disease states [7].

Since PCOS is a highly complex and heterogeneous disorder with a significant influence from environmental and genetic factors, no satisfactory theory still explains the clinical and biochemical diversity of the disease. Furthermore, despite having similar biochemical and hormonal similarities, some PCOS patients experience insulin resistance, accompanying autoimmune disease, and low oocyte fertilization to the implant, while others do not. Genetic differences among alleles at different genetic loci contributing to IR and autoimmunity are numerous possible explanations for this observation [8]. In this context, genetic aberrations that include microsatellites, single nucleotide polymorphisms (SNPs), and cell adhesion molecules can produce susceptibility to distinct disease states, including PCOS. Therefore, to investigate the processes linked with IR, inflammation, and genetic aberrations in PCOS, we designed the current study to ascertain the frequency of CTLA4+rs231775 polymorphism in Turkish PCOS women and its influence on clinical, biochemical, and hormonal phenotype.

Material and Methods

Study design

Ethical approval granted from Çanakkale Onsekiz Mart University local ethics committee (Ethical approval no: 2017/09). This cross-sectional study was performed in the Gynecology and Obstetrics department of Çanakkale Onsekiz Mart University Training and Research Hospital. Informed consent was obtained from all individuals who volunteered to participate in this study.

Subjects

PCOS diagnosis was made depending on the existence of predetermined criteria suggested by the Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group [9]. Exclusion criteria were defined as patients without any previous systemic, metabolic, or endocrine diseases. Furthermore, a history of oral contraceptive agent use, severe dyslipidemia, prescription of corticosteroids, or glucose-lowering drugs within the previous 90 days was also excluded. The healthy control group comprised 88 age and BMI-matched women with no systemic or endocrine diseases.

Laboratory analyses

An 8-ml fasting serum for hormonal and biochemical evaluation and 2 ml blood for genotyping were taken from each study participant after 8-12 hours of fasting during the early follicular phase of menstruation. The samples for biochemical analysis were further centrifuged at a speed of 6,000 rpm for 12 minutes at 4°C to obtain serum and saved for final analysis at -60 °C. Routine laboratory work-up and hormone tests, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), fasting glucose and insulin level, serum estradiol, total testosterone, prolactin, and thyroid-stimulating hormone (TSH) were measured for each subject. Biochemical tests were analyzed spectrophotometrically, and hormone tests were measured by the electrochemiluminescence immunoassay method. HOMA-IR estimated IR by using serum glucose and insulin levels.

Genotyping

The genomic DNA purification from mononuclear cells was performed using a commercial kit (Thermo Fisher Scientific, USA). Real-time polymerase chain reaction (PCR) was performed for each sample in a total volume of 10 μL PCR reaction mixture that consisted of 50 ng of genomic DNA, 5 μL of SYBR® Green Realtime PCR Master Mix (Analytic-Jena, Germany), and 0.4 μL of each primer (10 pmol/μL) filled with PCR-grade water. The PCR cycling protocol included initial activation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 20 seconds, and extension at 72°C for 15 seconds. Melting curve analyses following PCR were performed to determine mutant, heterozygous, and wild-type genotypes.

TNF-α and IL-6 measurement

TNF-α and IL-6 levels were determined with an enzyme-linked immunosorbent assay (ELISA) kit (TNF-α, Cat. No: KAP1751; IL-6 Cat KAP1261, DIAsource, Belgium). Results were shown as picograms per milliliter (pg/ml) of serum. The intra-assay and inter-assay coefficients of variations were < 6.6 and < 4.5, and < 4.3% and < 5.4 for TNF-alpha and IL-6, respectively.

Statistical analyses

Statistical Package for Social Sciences (SPSS) v20.0 (SPSS for Windows, SPSS, Chicago) was used for statistical analysis. Laboratory and hormonal parameters are demonstrated as mean ± SD for normally distributed variables, whereas non-normally distributed variables are presented as median (minimum-maximum). One-way analysis of variance (ANOVA) was used to compare normally distributed variables, whereas the Kruskal-Wallis test was used to compare non-normally distributed variables. Spearman's test was used for correlation analysis. A p-value below 0.05 was considered statistically

significant. The gene-counting method was used to measure allele and genotype frequencies, and comparisons within each subgroup were made with the chi-square test and Fisher's exact test. Genotype associations and relative risks were assessed via odds ratio by performing the Armitage trend test.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

A total of 92 PCOS women (mean age ± SD: 26.4 ± 6.1 years, range 17-46 years) and 88 healthy women (mean age ± SD: 28.9 ± 7.3 years, range 19-48 years) were studied in this trial. Overall, the mean BMI levels of PCOS patients and controls were 25.8 ± 5.2 and 24.0 ± 4.3, respectively. The detailed clinical features of the patients and control group are shown in Table 1. Although the demographic and clinical characteristics of PCOS women and healthy control group at the baseline with respect to age and BMI were found to be similar in both study groups, mean BMI levels of women with IR PCOS were statistically higher than those of non-IR PCOS patients and controls, as displayed in Table 1.

A statistically significant elevation was detected in body weight and insulin levels in PCOS women with HOMA-IR ≥ 2.5 compared to women with HOMA-IR below 2.5. Furthermore, patients with PCOS had a significant increase in respect to TNF-α, LH, and total testosterone levels (Figure 1) compared to controls. IL-6 levels were detected to be similar in PCOS women with HOMA-IR < 2.5, HOMA-IR ≥ 2.5, and controls (27.1 ± 10.5, 29.9 ± 19.4, and 27.7 ± 10.5 respectively) (Figure 2).

To analyze the further effect of obesity on HOMA-IR, TNF-α, IL-6, and other metabolic and hormonal parameters, we divided PCOS patients and controls into two groups according to BMI levels. As expected, HOMA-IR levels were shown to be elevated in PCOS women compared with healthy controls with respect to BMI levels (< 25 vs. ≥ 25 kg/m2). Although TNF-α levels were found to be elevated (p = 0.001) in PCOS women with BMI ≥ 25 kg/m2 compared to healthy controls with BMI ≥ 25 kg/m2, no difference was found between PCOS women and healthy controls with BMI < 25 kg/m2. Although increased BMI levels in PCOS women were found to be associated with an increasing trend in IL-6 levels, this was not statistically significant (Table 1).

When we investigated potential associations between TNF-α and IL-6 with respect to other demographic and clinical characteristics, we found that in the non-IR PCOS group, TNF-α levels were positively correlated with BMI (r=0.365, p=0.010) and HOMA-IR (r=0.335, p=0.019). However, in the IR PCOS group, TNF-α levels were correlated with only BMI levels (r=0.402, p=0.008).

The genotype distributions and carriage rate of CTLA4 promoter region polymorphisms in PCOS patients and controls are presented in Table 2. Genotype distribution of PCOS patients was as follows: out of 92 cases, 26 had wild (AA) genotype, 56 had heterozygous (AG), and 10 had mutant genotype (GG). The variant allele frequency (G allele) for CTLA4 + rs231775 polymorphism was calculated as 0.41 among PCOS patients, and the PCOS population was found to deviate from the Hardy-Weinberg equilibrium (HWE) (x^2:5.99; p-value:0.0143).

In contrast, the variant allele frequency (G allele) for CTLA4 + rs231775 polymorphism was calculated as 0.26, and none of the variants were found to deviate from the HWE in the control group (x^2:3.93; p-value:0.994).

The distribution of genotypes in the healthy controls was as follows: out of 88 controls, 48 had wild (AA), 34 had heterozygous (AG), and 6 had mutant genotype (GG). The frequency of the G-allele carriers on rs231775 was significantly elevated in patients with PCOS compared to control subjects (41% vs. 26% respectively). The present data indicate that the conversion of an A allele to a G in PCOS cases (allele frequency differences) increases PCOS risk 1.99 times compared to the general population (OR:1.989; CI=[1.273-3.107]; x^2=9.24, p=0.0024) (Table 2).

The AA genotype was related to a significantly elevated risk of PCOS development compared with the AG genotype (OR, 3.041; 95%CI, 1.60-5.77; x^2:11.92, p:0.0005). In this study, PCOS risk increases approximately three times compared to homozygous wild and mutant genotype [OR, 3.077; 95% CI, 1.005-9.421; x^2:4.10, p:0.043]. Allele positivity was found to significantly affect the increased risk of PCOS, namely three times greater than in counterparts with the AA wild genotype (OR:3.046;
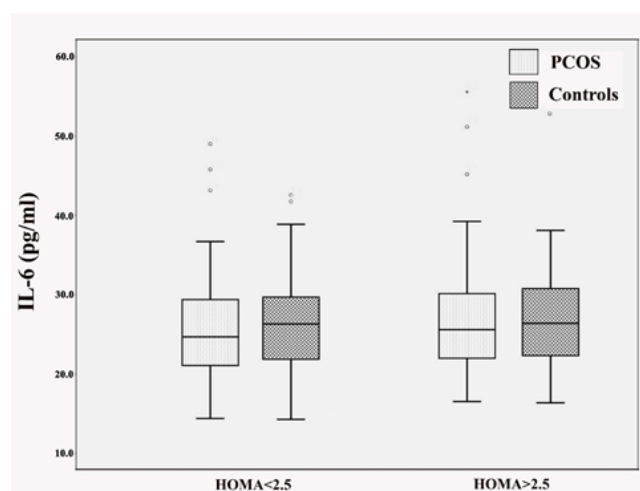

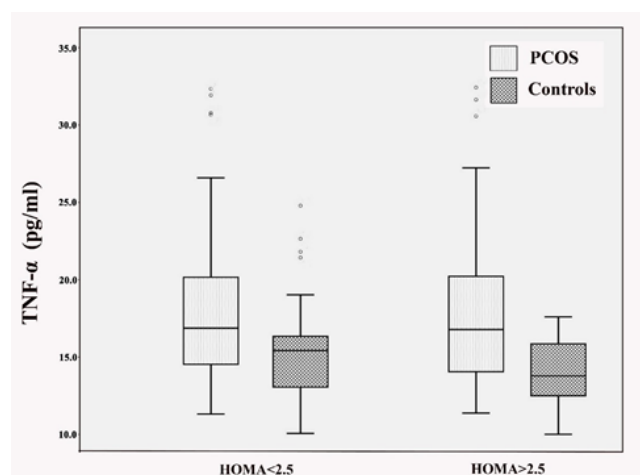**Figure 1.** Figure 1b. Correlation between serum IL-6 level with PCOS depending on HOMA-IR status.

Figure 2. Correlation between serum TNF-alfa level with PCOS depending on HOMA-IR status.

Table 1. Demographic and laboratory characteristics of PCOS women and healthy controls and Serum levels of TNF-α, IL-6 and CRP with other demographic and hormonal parameters according to BMI levels.

	PCOS (n=92)		CG	p	BMI <25			BMI≥25		
	HOMA-IR									
	<2.5	≥2.5			PCOS (n=44)	CG (n=57)	p	PCOS (n=48)	CG (n=31)	p
	(n=49)	(n=43)	(n=88)							
Age (years)	27.5 ±6.1	25.1 ±5.3	28.8 ±7.3	NS						
Glucose (mg/dl)	91.5 ±12.6	96.1 ±14.7	93.7 ±17.2	NS	25.6 ±5.3	27.4 ±6.6	NS	27.1 ±6.8	31.7 ±7.9	0.008
Insulin (µIU/ml)	6.8 ±2.7	19.3 ±9.2	8.3 ±5.5	0.018c						
HOMA-IR (%)	1.5 ±0.6	4.4 ±2.1	1.9 ±1.3	0.001d	2.2 ±1.7	1.7 ±1.2	0.04	3.4 ±2.2	2.4 ±1.4	0.020
TSH (µIU/ml)	2.4 ±1.2	2.4 ±0.9	2.3 ±1.2	NS	2.3 ±1.1	2.2 ±1.2	NS	2.4 ±1.0	2.5 ±1.2	NS
FSH (mIU/ml)	6.1 ±2.1	5.4 ±1.5	5.3 ±2.2	NS	5.9 ±1.9	5.0 ±2.1	0.02	5.8 ±1.8	5.9 ±2.5	NS
LH (mIU/ml)	7.7 ±2.9	8.0 ±2.8	6.3 ±3.8	0.022e	7.8 ±2.7	6.1 ±3.8	0.02	7.9 ±3.1	6.6 ±4.1	0.014
TT (pg/ml)	0.32 ±0.15	0.46 ±0.18	0.29 ±0.13	0.001f	0.35 ±0.2	0.30 ±0.12	NS	0.42 ±0.2	0.28 ±0.2	<0.001
Estradiol (pg/ml)	49.4 ±26.1	46.4 ±23.6	53.5 ±30.4	NS	48.8 ±26.7	55.0 ±32.9	NS	47.3 ±23.0	50.8 ±25.4	NS
Prolactin (ng/ml)	22.0 ±11.2	23.2 ±11.4	20.1 ±12.5	NS	22.6 ±12.1	21.5 ±13.6	NS	22.6 ±10.0	17.2 ±9.7	0.03
TNF-α (pg/ml)	18.3 ±5.3	18.0 ±5.3	14.8 ±2.8	0.001g	16.3 ±4.3	15.0 ±2.6	NS	20.0 ±5.6	14.3 ±3.2	<0.001
IL-6 (pg/ml)	27.1 ±10.5	29.9 ±19.4	27.7 ±10.5	NS	24.0 ±6.4	28.7 ±12.2	0.02	32.5 ±19.6	25.9 ±6.2	NS
Hs-CRP	0.37 ±0.41	0.44 ±0.47	0.42 ±0.60	NS	0.4 ±0.5	0.3 ±0.6	NS	0.4 ±0.3	0.5 ±4.0	NS

a,b,c,d PCOS HOMA-IR≥ 2.5 versus PCOS HOMA-IR<2.5 and controls; e, f ,g PCOS HOMA-IR ≥ 2.5 and PCOS HOMA<2.5 versus controls, C:Controls, NS: not significant; BMI: body mass index; HOMA-IR: homeostasis model assessment of insulin resistance; TSH: thyroid stimulating hormone; FSH: follicle stimulating hormone; LH: luteinizing hormone; TT: total testosterone; TNF-α: tumor necrosis factor-α; IL-6:interleukin-6; hs-CRP: high-sensitivity C-reactive protein

Table 2. Distributions of genotype and carriage rate of CTLA4 promoter region polymorphisms (rs231775) in patients with PCOS and healthy controls (upper part) and distributions of genotype and carriage rate of CTLA4 promoter region polymorphisms (rs231775) in PCOS patients according to HOMA-IR status (lower part).

SNP	Tests for deviation from Hardy-Weinberg equilibrium		Tests for association (95% CI)				
	Controls	PCOS	allele freq. difference	heterozygous	homozygous	allele positivity	Armitage's trend test
CTLA4 rs231775	nAA=48 (47.63)	nAA=26 (31.70)	Risk allele G				
	nAG=34 (32.74)	nAG=56 (44.61)	[A]<->[G]	[AA]<->[AG]	[AA]<->[GG]	[AA]<->[AG+GG]	Common OR
	nGG=6 (5.63)	nGG=10 (15.70)	OR=1.989	OR=3.041	OR=3.077	OR=3.046	OR=1.975
	f_a1=0.74 +/-0.033	f_a1=0.59 +/-0.031	C.I.=[1.273-3.107]	C.I.=[1.604-5.766]	C.I.=[1.005-9.421]	C.I.=[1.642-5.652]	x^2: 10.45
	F=-0.00067	F=-0.25536	x^2: 9.24	x^2: 11.92	x^2: 4.10	x^2: 12.84	p=0.00123
	p=0.994993 (Pearson)	p=0.014312 (Pearson)	p=0.00237 (P)	p=0.00056	p=0.04276	p=0.00034	
	p=0.994993 (Llr)	p=0.013104 (Llr)					
	p=1.000000 (Exact)	p=0.018885 (Exact)					
CTLA4 rs231775	HOMA-IR<2.5	HOMA-IR>2.5	[A]<->[G]	[AA]<->[AG]	[AA]<->[GG]	[AA]<->[AG+GG]	Common OR
	nAA=13 (14.33)	nAA=13 (16.33)	Odds_ratio=0.733	Odds_ratio=1.000	Odds_ratio=0.333	Odds_ratio=0.833	Odds_ratio=0.640
	nAG=27 (24.34)	nAG=27 (20.34)	C.I.=[0.407-1.321]	C.I.=[0.392-2.549]	C.I.=[0.073-1.518]	C.I.=[0.336-2.068]	x^2: 1.34
	nGG=9 (10.33)	nGG=3 (6.33)	x^2: 1.07	x^2: 0.00	x^2: 2.11	x^2: 0.15	p=0.24738
	f_a1=0.54 +/-0.048	f_a1=0.62 +/-0.043	p=0.30138 (P)	p=1.00000	p=0.14681	p=0.69398	
	F=-0.10943	F=-0.32762					
	p=0.443654 (Pearson)	p=0.031688 (Pearson)					
	p=0.442946 (Llr)	p=0.027018 (Llr)					
	p=0.569243 (Exact)	p=0.053417 (Exact)					

Table 3. Serum TNF-α, IL-6 and BMI levels of PCOS patients and controls with different genotypes of CTLA4+ rs231775 polymorphism

	PCOS patients (n=92)							p
	AA (n=26)	AG (n=56)	GG (n=10)	p	AA (n=48)	AG (n=34)	GG (n=6)	
TNF_Alpha (pg/ml)	17.8 (12.4-32.4)	15.9 (11.3-32.5)	19.6 (12.4-24.9)	0.217	15.1 (10.0-22.6)	14.7 (10.0-24.8)	13.6 (11.3-17.6)	0.760
IL_6 (pg/ml)	24.6 (14.5-142.3)	25.2 (16.5-77.3)	25.3 (17.7-39.2)	0.977	26.6 (14.3-92.8)	26.7 (15.7-53.5)	22.3 (16.7-33.8)	0.394
BMI (kg/m²)	27.4 (16.8-42.5).	24.5 (16.1-37.6)	24.7 (19.3-29.0)	0.244	24.1 (16.6-36.7)	23.0 (17.0-33.6)	23.6 (20.2-27.5)	0.920
HOMA-IR	2.5 (0.6-8.3)	2.5 (0.4-12.9)	1.8 (0.4-4.9)	0.318	1.3 (0.4-5.2)	1.9 (0.4-5.6)	2.3 (0.9-4.7)	0.066

TNF-α: tumor necrosis factor- α; IL-6: interleukin-6; BMI: body-mass index; HOMA-IR: homeostasis model assessment of insulin resistance

95%CI = [1.642-5.652], x^2:12.84; p=0.0003). A strong allelic (with G as the risk allele) or genotypic (GG or AG) association was found for CTLA4+rs231775 polymorphism in the PCOS group.

The same genotypic analysis was also executed after PCOS women were divided into two subgroups according to HOMA-IR status (Table 3). No significant relation was found between CTLA-4+rs231775 polymorphism and HOMA-IR status among the PCOS group.

In patients with PCOS, there was no significant difference in the TNF-α and IL-6 levels in carriers of the genotypes AA, AG, and GG of CTLA-4+rs231775 polymorphism. In the healthy control group, the TNF-α and IL-6 levels in carriers of genotypes AA, AG, and GG of CTLA-4+rs231775 polymorphism were also not significantly different. We also explored the genotypic differences in PCOS patients concerning IR status. In patients with HOMA-IR<2.5, the TNF-α and IL-6 levels did not differ significantly in carriers of AA, AG, and GG genotypes of CTLA-4+rs231775 polymorphism. In PCOS patients with HOMA-IR ≥2.5, there were only two individuals with the GG genotype. Neither of these individuals was included in the final analysis. Again, no significant difference was found in this subgroup between carriers of the AA and AG genotypes of CTLA-4+rs231775 polymorphism.

Discussion

Overstimulation of the immune system in PCOS usually alters the production and secretion of inflammatory cytokines and leads to various clinical and metabolic manifestations [10, 11]. The present study's findings demonstrate that serum TNF-α levels in PCOS women (either with the presence or absence of IR) are elevated compared with controls. On the other hand, IL-6 levels were comparable in PCOS women and controls, independent of IR status. One interesting finding of the present study is the correlation between TNF-α levels with BMI. We demonstrated that only in obese (BMI≥25 kg/m2) PCOS patients serum TNF-α levels were elevated compared with controls. In this context, our findings are consistent with previous research studies applied in obese and non-obese PCOS women except for the unaltered IL-6 levels [11, 12].

The polymorphic sites in the CTLA4 gene, including C>T polymorphism in the promoter -318 (rs5742909) and A>G polymorphism in exon 1 +49A/G (rs231775), are demonstrated to be linked with a variety of autoimmune diseases [13-15]. Among these, rs231775 is the most extensively investigated immune disorder marker [16, 17]. Based on the altered immune system in PCOS pathophysiology, we hypothesized that CTLA4 rs231775 polymorphism might be associated with PCOS development.

In the present study, we also demonstrated a significant alteration in the distribution of CTLA4+rs231775 A/G mutant allele frequency between patients and controls. The genotype-phenotype association revealed that the G allele of SNP rs231775 was more frequent in PCOS women than in healthy controls (OR:1.99, 95%CI:1.273-3.107, p=0.0023). The heterozygous genotype was also shown to be strongly associated with PCOS development (OR:3.041, 95% CI:1.604-5.766, p=0.00056). The excess of heterozygous (AG) mutant

variants and G alleles observed in our patients may indicate a susceptibility to developing PCOS regardless of IR in PCOS. The association between PCOS and IL-6 and TNF-α is well known, but the relation between CTLA4+rs231775 gene polymorphism with these parameters has not been studied. Therefore, we determined whether IL-6 and TNF-α levels were altered in PCOS women and healthy volunteers genotyped with CTLA4+rs231775 gene polymorphism. Although no data in the literature provide evidence for a possible immunologic mechanism underlying CTLA4 gene polymorphism, TNF-α, and IL-6 in PCOS patients, various reports are exploring the intimate relationship between these parameters in distinct disease states. Han et al. [18] demonstrated that the CTLA4+49 GG genotype was related to TNF-α and IFN-gamma levels in HBV-infected patients, and this association was found to be decreased by haplotype formation with -318C/T alleles.

Conclusion

In conclusion, our results suggest strong evidence of the relationship between circulating TNF-α and PCOS. BMI and IR were the parameters most strongly associated with TNF-α levels. Enhanced levels of TNF-α in PCOS (particularly in women with PCOS-IR and obese PCOS women) may represent a significant underlying factor in the diverse clinical manifestations seen in patients with PCOS. Moreover, we also demonstrated for the first time that CTLA4+rs231775 gene polymorphism is strongly associated with PCOS development, providing advances in understanding the molecular basis of PCOS development independent from BMI, HOMA-IR, TNF-alpha, and IL-6 levels in the clinic. Although, CTLA4+ rs231775 polymorphism is a potential genetic risk factor for PCOS and is not affected by insulin resistance or increased body weight. As this is the first report on the relation between CTLA4+rs231775 polymorphism and PCOS, further trials will be required to outline the role of genetic polymorphism on the expression and functional properties of CTLA4.

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